SHOCK, Vol. 48, No. 3, pp. 313–320, 2017

HEPARIN-BINDING PROTEIN (HBP): A CAUSATIVE MARKER AND POTENTIAL TARGET FOR HEPARIN TREATMENT OF HUMAN SEPSIS-INDUCED ACUTE KIDNEY INJURY

Jane Fisher,[*†] James A. Russell,[†] Peter Bentzer,[†‡§] Devyn Parsons,[†] Stefano Secchia,[*] Matthias Mörgelin,[*] Keith R. Walley,[†] John H. Boyd,[†] and Adam Linder[*†]

[*]Department of Infectious Diseases, Lund University and Skåne University Hospital, Lund, Sweden; [†]Centre for Heart Lung Innovation, Division of Critical Care Medicine, St. Paul's Hospital, University of British Columbia, Vancouver, BC, Canada; [‡]Department of Anesthesia and Intensive Care, Helsingborg Hospital, Helsingborg, Sweden; and [§]University of Lund, Lund, Sweden

Received 3 Jan 2017; first review completed 20 Jan 2017; accepted in final form 24 Feb 2017

ABSTRACT—**Rationale:** Sepsis-induced acute kidney injury (AKI) is a common condition with high morbidity and mortality. Neutrophil-derived heparin-binding protein (HBP) induces vascular leakage and is a promising biomarker of sepsis-induced organ dysfunction. It remains unknown if HBP is prognostic of AKI in septic shock and if HBP could play a role in the pathophysiology of sepsis-induced AKI. **Objectives:** To determine the association of plasma HBP levels with development of AKI, investigate the role of HBP in the pathophysiology of sepsis-induced AKI, and test the effect of blocking HBP using heparin derivatives. **Methods:** In 296 septic shock patients from the randomized multicenter Vasopressin and Septic Shock Trial (VASST) plasma HBP levels were associated with development of AKI and need for renal replacement therapy (RRT). Human renal tubular cells were exposed to recombinant HBP to evaluate inflammation and heparin derivatives were used to abrogate these effects. Finally, mice were exposed to HBP with and without heparin derivatives and the kidneys examined for signs of inflammation. **Findings:** Plasma HBP levels were significantly higher in patients with AKI and those requiring RRT. HBP levels identified patients with moderate AKI with an area under curve (AUC) of 0.85. HBP increased IL-6 production in renal tubular epithelial cells. Different heparin derivatives abrogated the HBP-induced increased inflammatory response *in vitro* and *in vivo*. **Conclusion:** Elevated plasma HBP is associated with development of sepsis-induced AKI and HBP is involved in its pathophysiology. Our studies suggest that heparin(s) could be tested for efficacy and safety of prevention of sepsis-induced AKI.

KEYWORDS—Acute kidney injury, heparin derivatives, heparin-binding protein (HBP), infection, septic shock

ABBREVIATIONS—AKI—acute kidney injury; HBP—heparin-binding protein; ICU—intensive care unit; LMWH—low molecular weight heparin; RRT—renal replacement therapy; UFH—unfractionated heparin

INTRODUCTION

Patients who develop acute kidney injury (AKI) in septic shock have higher short- and long-term mortality (1–3) than those who do not. The 2012 Kidney Disease: Improving Global Outcomes (KDIGO) Guidelines highlight the need for accurate assessment of increasing severity of AKI after renal injury (4). Novel AKI biomarkers diagnose AKI prior to increased creatinine (5, 6), but lack the sensitivity to predict worsening AKI and the need for renal replacement therapy (RRT) (7). Sepsis-induced AKI has commonly been attributed to acute tubular necrosis and apoptosis (8). Recently, a combination of vascular leakage/hypoperfusion, local renal tubular inflammation, and cell cycle arrest has been suggested to mediate sepsis-induced AKI (9).

Heparin-binding protein (HBP, also known as azurocidin or CAP37), stored by neutrophils, is released early upon neutrophil adhesion and extravasation (10, 11). Bacterial products induce HBP release (12) and HBP is the key mediator of neutrophil-dependent vascular permeability increases (13, 14). Elevated plasma levels of HBP are a promising early biomarker of severe sepsis in the emergency department (15, 16) and correlate with the development of septic shock (17). Whether HBP induces renal tubular cell inflammation or cell cycle arrest is unknown. Also to date, there have been no studies of the association of HBP with AKI in general.

Heparin is a multifunctional negatively charged glycosaminoglycan (18) that binds to HBP (19). Heparin derivatives may be beneficial in sepsis treatment (20) and mitigate progression to AKI in a murine sepsis model (21). Because heparin has

Address reprint requests to Adam Linder, MD, PhD, Department of Infectious Diseases, Lund University and Skåne University Hospital, Lund, Sweden; E-mail: adam.linder@med.lu.se

AL is listed as an inventor on a pending patent application on the use of HBP as a diagnostic tool in sepsis.

Author contributions: Conception and design: AL, JF, PB, JHB, JAR. Acquisition of data: JAR, KRW, PB, JHB, JF. Analysis and interpretation of data: DP, JF, SS, MM, AL, JAR, JHB, PB. Drafting or revising the article: DP, JF, AL, JAR, JHB, KRW, PB. Final approval of the manuscript: DP, JF, PB, MM, SS, JAR, JHB, KRW, AL.

Support for current study: Canadian Institutes of Health Research (CIHR) (SONRIS) (JAR). Swedish Government Research Grant (ALF) (AL, PB), Svenska läkare sällskapet (AL), SUS fonder (AL) Svenska Vetenskapsrådets Post-doctoral grants (AL), CIHR IMPACT strategic training Post-doctoral Fellowship (AL), Groschinsky foundation (AL), Lundgrens foundation (AL), Österlunds foundation (AL), Åke Wiberg foundation (AL), Providence Health Care Research Scholarship (JHB), The Anna and Edwin Berger Foundation (PB). The commercial HBP ELISA used in this study was kindly provided by Axis-Shield Diagnostics, Dundee, UK.

The authors report no conflicts of interest other than those stated for AL above.

Supplemental digital content is available for this article. Direct URL citation appears in the printed text and is provided in the HTML and PDF versions of this article on the journal's Web site (www.shockjournal.com).

DOI: 10.1097/SHK.0000000000000862

many biological effects apart from anticoagulant activity, including anti-inflammatory properties, it is a potentially interesting therapeutic option in sepsis.

Accordingly, we hypothesized that elevated plasma HBP levels are associated with sepsis-induced AKI. Second, we tested whether HBP induces renal inflammation and investigated potential mechanisms mediating this effect. Third, we tested if heparin derivatives could block HBP *in vitro* and *in vivo.*

PATIENTS AND METHODS

Patient selection and definitions

Plasma samples were obtained from the Vasopressin and Septic Shock Trial (VASST) cohort of septic shock patients (22). Approval, enrollment, and consent in the VASST trial have been described previously (22). Patients receiving chronic renal replacement therapy (RRT) were excluded, leaving 296 patients with baseline plasma samples. At least two consecutive serum creatinine (sCr) measurements over the first 5 days were available from 284 (96.7%) patients and 257 (86.6%) patients had 5 or more sCr measurements available.

The primary clinical outcome investigated was development of any stage AKI over the first 5 days. AKI was staged according to the KDIGO classification guidelines using sCr (4). The lowest sCr in the 5 days following plasma sample collection was defined as baseline and the magnitude of change in sCr anytime in the first 5 days was used to classify AKI stage. A secondary outcome was needed for acute RRT. Development of coagulation (platelets $\leq 80 \times 10^3/mm^3$), hepatic (bilirubin ≥ 2.0 mg/dL), and central nervous system dysfunction (Glasgow score ≤ 12) were defined as a progression to a moderate Brussels score within the first 28 days following study enrollment.

Laboratory analysis

Plasma samples—Plasma HBP concentration was measured in duplicate using a commercial HBP ELISA (Axis-Shield Diagnostics, Dundee, UK) according to the manufacturer's directions. Analyses were performed with positive and negative controls and blinded to clinical outcomes.

Interleukin-6 (IL-6) in plasma was measured at baseline and at 24 h as part of a Luminex multiplex cytokine panel. Concentrations were converted to pM as described previously (23, 24) and log-transformed.

Renal cell culture—HK-2 cells (American Type Culture Collection, Manassas, Va), a proximal tubular epithelial cell line, were cultured in keratinocyte serum-free media supplemented with bovine pituitary extract and epidermal growth factor 1–53 (Life Technologies, Thermo Fisher Scientific Inc., Waltham, Mass). Cells were stimulated with HBP and/or inhibitors in supplemented media.

HBP-induced renal inflammation—HK-2 cells were cultured as above and stimulated with 0, 1, and 10 μg/mL of HBP (R&D Systems). After 6, 24, and 48 h, supernatant was collected and analyzed by a Luminex multiplex quantitation assay (R&D Systems Minneapolis, Minn). Cytokines analyzed were ICAM-1, IL-6, TNFα, IL-10, and VEGF. IL-6 was elevated in response to HBP and thus chosen as a marker of HBP-induced inflammation for all further *in vitro* experiments and was measured after 24 h of stimulation with 0 or 10 μg/mL HBP using the IL-6 DuoSet ELISA (R&D Systems) according to the manufacturer's directions.

Signaling pathway inhibitors—HK-2 cells were pre-incubated with signaling pathway inhibitors for 1 h and stimulated with HBP, and supernatant IL-6 levels were measured. The inhibitors used were RO 31-8220 (Tocris, 100 nM, Minneapolis, Minn) and Calphostin C (Tocris, 50 nM) to inhibit Protein Kinase C (PKC), pyrrolidine dithiocarbamate (PDTC) (Sigma Aldrich, 30 μM, St. Louis, Mo) to inhibit interferon kappa-B alpha (IκBα) degradation, BIX 02189 (Tocris, 30 μM) to inhibit mitogen-activated protein kinase kinase 5 (MEK5), JNKi plus control peptide (Millipore, 10 μM, Darmstadt, Germany) to inhibit c-jun N-terminal Kinase (JNK), CI-1040 (Selleckchem, 10 μM, Houston, Tex) to inhibit MEK1/2, and SB202190 (Millipore, 10 μM) to inhibit P38 mitogen-activated protein kinase (MAPK).

Heparins as inhibitors of HBP—The heparin compounds used in this study were unfractionated heparin (UFH, Leo) and three low molecular weight heparins (LMWH): Dalteparin (Fragmin, Pfizer), Enoxaparin (Klexane, Sanofi Aventis), and Tinzaparin (Innohep, Leo). Therapeutic plasma UFH levels occur between 0.3 and 0.7 anti-Xa Units/mL (25). Therapeutic LMWH levels vary between 0.6 and 1.3 anti-Xa Units/mL (26). UFH and all LMWH were used at a concentration of 1 Unit/mL. HBP was pre-incubated with or without the indicated heparin at 37°C for 20 min. Cells were stimulated with this mixture and IL-6 levels were measured.

Glycosaminoglycan digestion

Since HBP binds to proteoglycans via glycosaminoglycan (GAG) moieties (27), we examined whether cell surface GAGs are required for HBP-induced IL-6 production. HK-2 cells were treated with 15 mU/mL of Heparinase III (New England Biolabs, Ipswich, Mass) or 2.5 mU/mL Chondroitinase ABC (R&D Systems) for 1 h at 37°C (28). Cells were stimulated with HBP and IL-6 levels were measured.

In vivo experiments

Anaesthetized mice (see Protocol—Animal Experiments, Supplemental Digital Content 1, http://links.lww.com/SHK/A571) received one of four treatments: bolus intravenous (i.v.) HBP (100 μg) followed by HBP infusion (2.5 μg/g/h for 1 h) (n = 3), bolus i.v. UFH (0.4 E/g) (29) followed by i.v. HBP (100 μg) followed by HBP infusion as above (n = 3), bolus i.v. vehicle (100 μL 0.9% normal saline) followed by vehicle infusion (1.25 μL/g/h) (n = 5), intraperitoneal injection of Lipopolysaccharide from *Escherichia Coli* 0111:B4 (LPS, Sigma-Aldrich) (0.25 mg) (30). One hour after treatments 1 to 3 or 4 h after treatment 4, the animals were killed by exsanguination.

Electron microscopy, histology, and immunohistochemistry

Kidneys were prepared for histology, immunohistochemistry with anti-albumin antibody, and electron microscopy. Images were acquired and staining was quantified when applicable (see Protocol—Animal Experiments, Supplemental Digital Content 1, http://links.lww.com/SHK/A571). All analyses were performed in a blinded manner.

Ethics

The VASST study was approved by the research ethics boards of all participating institutions (22). The local Ethical Committee for Animal Research (M80-14) approved the protocol for animal experiments. Animals were treated in accordance with the National Institutes of Health for the Care and Use for Laboratory animals.

Statistical analysis

Patient data were analyzed using GraphPad Prism (version 7.0) and SPSS (version 19.0). Specific statistical tests used are described in the figure legends. Receiver operator characteristic (ROC) curves were used to confirm the association of HBP with the development of AKI. Cases were selected as those who developed stage 2 AKI and controls were those who did not develop AKI, and the whole range of measured HBP levels was used to generate the curves. Adjusted analyses were done using a logistic regression model.

In vitro data were analyzed in GraphPad Prism with all values presented as the mean and standard error of the mean (SEM). *P* values less than 0.05 were considered significant.

RESULTS

Demographics

The 296 patients analyzed had a mean age of 60.1 years and 58.6% males (Table 1). Of these patients, 225 (76.0%) patients had or developed AKI of any stage within 28 days: 71 patients never had AKI (24.0%), 123 patients had AKI stage 1 (41.6%), 67 patients (22.6%) had stage 2, and 35 patients (11.8%) had stage 3. There were no differences in age, gender, and ethnicity between these subgroups. However, patients with any stage of AKI had higher mean APACHE II scores, less lung infections, and higher prevalence of positive blood cultures with gram negative bacteria (Table 1).

Elevated plasma HBP levels are associated with development of AKI

HBP levels were significantly higher in patients with any stage AKI (median 38.9 ng/mL [IQR 18–98]), and with different AKI stages, than in those with no AKI (9.5 ng/mL (4–23)) ($P < 0.01$, Fig. 1A). ROC analysis confirmed that HBP could

TABLE 1. **Patient demographics**

Characteristic	No AKI (n=71)	Any AKI (n=225)	*P* value	Stage 1 (n=123)	Stage 2 (n=67)	Stage 3 (n=35)
Male, n (%)	42 (59.2)	132 (58.6)	1.00	70 (56.8)	37 (55.2)	25 (71.4)
Age, yr mean (SD)	60.2 (15.7)	59.0 (17.2)	0.68	60.3 (16.6)	58.1 (18.5)	56.6 (16.7)
Caucasian, n (%)	72 (94.7)	192 (87.3)	0.74	103 (87.3)	55 (82.1)	34 (97.1)
APACHE II, mean (SD)	21.7 (6.7)	27.1 (7.8)	<0.01	25.8 (7.1)	28.8 (7.9)	28.0 (9.0)
Coagulopathy n (%)	15 (21.1)	107 (48.6)	<0.01	46 (37.4)	42 (62.7)	19 (54.3)
Infection site						
Lung, n (%)	43 (56.6)	88 (40.0)	0.02	50 (42.4)	24 (35.8)	14 (40.0)
Abdomen, n (%)	11 (14.5)	67 (30.5)	<0.01	37 (31.4)	20 (29.9)	10 (28.6)
Other, n (%)	22 (28.9)	65 (29.5)	1.00	31 (26.3)	23 (34.3)	11 (31.4)
Blood culture						
Gram+ bacteria, n (%)	21 (27.6)	63 (28.6)	1.00	30 (25.4)	21 (31.3)	12 (34.3)
Gram− bacteria, n (%)	8 (10.5)	49 (22.3)	0.03	28 (23.7)	15 (22.4)	6 (17.1)
Comorbidities						
CHF, n (%)	6 (8.4)	13 (5.8)	0.30	5 (4.1)	6 (9.0)	2 (5.7)
COPD, n (%)	22 (31.0)	24 (10.7)	0.02	15 (12.2)	7 (10.4)	2 (5.7)
Chronic steroids, n (%)	11 (14.5)	46 (20.9)	0.24	26 (22.0)	12 (17.9)	8 (22.9)
Chronic hepatic failure, n (%)	7 (9.2)	28 (12.7)	0.54	16 (13.6)	8 (11.9)	4 (11.4)

Groups were compared using the Student *t* test or Mann–Whitney test or Chi-squared test as appropriate.
AKI indicates acute kidney injury (defined by KDIGO classification); APACHE II, Acute Physiology and Chronic Health Evaluation II; CHF, congestive heart failure; COPD, chronic obstructive pulmonary disorder; SD, standard deviation.

differentiate between patients with stage 2 AKI and those without AKI with an area under curve (AUC) of 0.85 (0.79–0.91) ($P<0.01$, Fig. 1B). HBP remained significantly associated with the presence of any AKI stage ($P<0.01$) in a logistic regression model adjusting for age, gender, APACHE II, and comorbidities (chronic heart failure, COPD, chronic steroid treatment and chronic hepatic failure).

Plasma HBP levels were correlated with increased serum creatinine (not shown) and elevated plasma levels of HBP were associated with an increased risk of development of AKI after inclusion. We found that 100 patients did not have AKI in the first 48 h. Of these, 29 later developed AKI. The patients who developed AKI had significantly higher HBP levels than those who did not ($P<0.01$). A ROC curve of HBP levels

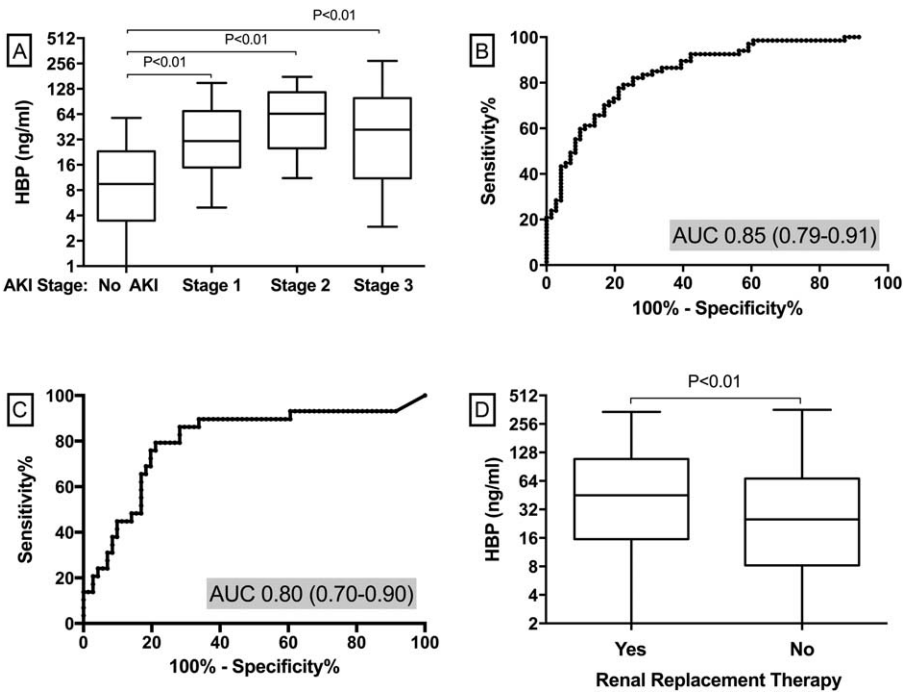

FIG. 1. **Association of HBP with AKI in human septic shock.** A, HBP levels in plasma of patients with various stages of AKI. Patients with stages 1, 2, or 3 of AKI had significantly higher HBP levels than those with no AKI ($P<0.01$, $P<0.01$, $P<0.01$). Groups were compared using a one-way ANOVA followed by Dunns post-test for multiple comparisons. B, Receiver operating characteristics curve showing that measuring HBP could identify patients with stage 2 AKI severity with a AUC of 0.85 (95% CI 0.79–0.91). C, Receiver operating characteristics curve showing that measuring HBP could identify patients who did not present with AKI within the first 48 h but later developed AKI with a AUC of 0.80 (95% CI 0.70–0.90). D, HBP levels were significantly higher among patients who received renal replacement therapy in the first 28 days, excluding patients with chronic dialysis (median HBP 45.1 ng/mL) compared with patients who did not receive RRT (median HBP 25.1 ng/mL) ($P<0.01$). AKI indicates acute kidney injury; AUC, area under curve; HBP, heparin-binding protein; RRT, renal replacement therapy.

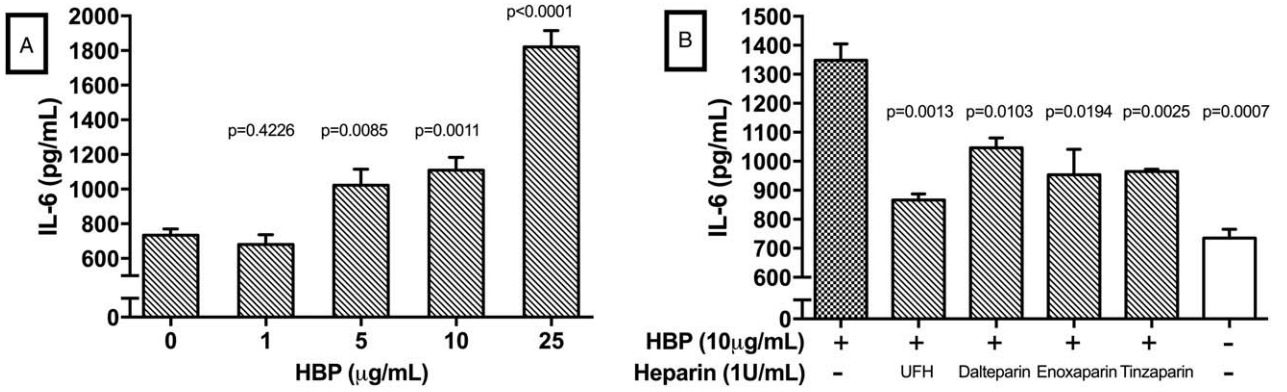

Fig. 2. **Heparin derivatives block IL-6 production by renal tubular epithelial cells.** A, HK-2 cells were stimulated with varying doses of HBP and the level of IL-6 was measured in the supernatant 24 h following stimulation. Groups were compared to stimulation with 0 μg/mL HBP (far left) using a one-way ANOVA followed by Dunns post-test for multiple comparisons. B, HBP (10 μg/mL) was pre-incubated with the indicated heparin prior to stimulation of HK-2 cells. IL-6 levels were measured in the supernatant 24 h following stimulation. Groups were compared to stimulation with HBP alone (far left) using a one-way ANOVA followed by Dunns post-test for multiple comparisons. Data are the mean IL-6 concentration n =3. HBP indicates heparin-binding protein; IL-6, interleukin-6.

differentiated these two groups with an AUC of 0.80 (0.70–0.90) (Fig. 1C). Finally, plasma HBP levels were significantly higher ($P < 0.01$) in patients who needed acute RRT compared with those who did not (Fig. 1D). HBP levels were also significantly correlated with plasma IL-6 levels at baseline and 24 h ($R = 0.29$ $P < 0.001$ and $R = 0.26$ $P < 0.001$ respectively, see Fig. 1A and B, Supplemental Digital Content 2, http://links.lww.com/SHK/A572).

HBP and other organ dysfunctions

HBP levels were significantly negatively correlated with platelet count for days 1–5 ($R \leq -0.19$, $P < 0.001$, see Table 1, Supplemental Digital Content 2, http://links.lww.com/SHK/A572) and were significantly higher in patients with coagulopathy (determined by the Brussels score) anytime within 28 days compared with those without (37.2 vs. 20.9 ng/mL, $P < 0.001$) and in patients with hepatic failure compared with those without (33.9 vs. 23.0, $P = 0.01$) (see Fig. 2, Supplemental Digital Content 2, http://links.lww.com/SHK/A572). HBP levels were not significantly associated with mean arterial pressure, central venous pressure or hepatic or central nervous system dysfunction (not shown). The association of HBP with respiratory dysfunction in this cohort of patients has been reported previously (14).

HBP increases renal tubular epithelial cell inflammation

Measurement of several cytokines in the supernatant of HBP-stimulated HK-2 cells by a Luminex multiplex quantitation assay indicated that IL-6 was a potential marker for renal cell inflammation (not shown). To confirm this result, cells were stimulated with varying doses of HBP and IL-6 was measured in the supernatant after 24 h. Concentrations of HBP above 5 μg/mL significantly increased IL-6 production (Fig. 2A). These results suggest that HBP induces inflammation of renal epithelial cells and the relationship between plasma HBP levels and AKI severity may indeed be causative. Therefore finding inhibitors to block HBP is of interest.

Heparins inhibit HBP-induced inflammation

The effect of heparin derivatives on HBP-induced renal cell inflammation was assessed by stimulating HK-2 cells with HBP, pre-incubated with UFH or one of three LMWH, and IL-6 was measured in the supernatant after 24 h. All four forms of heparin significantly decreased IL-6 production (Fig. 2B), suggesting that the particular LMWHs do not significantly differ in their ability to inhibit HBP-induced renal cell inflammation. The heparin concentrations used (1 Unit/mL) were within the therapeutic range for LMWH (0.6–1.3 Units/mL).

PKC and IκBα are involved in HBP-induced inflammation

To determine which signaling pathways are involved in HBP-induced IL-6 production, HK-2 cells were pretreated with signaling pathway protein inhibitors. The cells were then stimulated with HBP, and IL-6 was measured after 24 h. Pretreatment with PKC inhibitors Calphostin C and RO 31-8220 and IκBα degradation inhibitor PDTC, all blocked the HBP-induced increase in IL-6 (Fig. 3A). PDTC treatment decreased IL-6 production slightly below the level of the control, indicating that un-stimulated cells have a small background level of IκBα activity. The other inhibitors BIX 02189 (MEK5/ERK5), CI-1040 (MEK1/2), SB202190 (P38-MAPK), and JNKi (JNK) had no effect on HBP-induced IL-6 production (not shown). These results indicate that pathways involving PKC and IκBα are necessary for HBP-induced IL-6 production by renal tubular cells.

Cell-surface glycosaminoglycans are necessary for HBP-induced inflammation

Because HBP can bind to cell surface proteoglycans via their GAGs (27), we tested the hypothesis that GAGs are required for HBP-induced IL-6 production. Heparinase III or chondroitinase ABC were used to cleave heparan or chondroitin/dermatan sulfate respectively from the surface of HK-2 cells (31, 32). Treatment with heparinase III and chondroitinase ABC decreased HBP-induced IL-6 production (Fig. 3B), suggesting

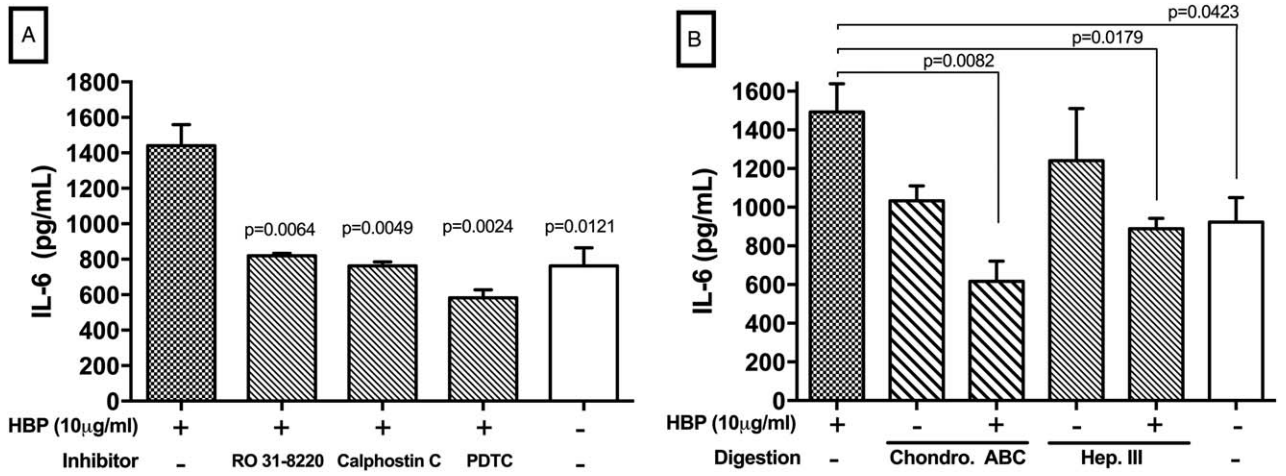

FIG. 3. **HBP-induced IL-6 production requires PKC and IκBα, and the presence of cell surface heparan and chondroitin/dermatan sulfate.** A, HK-2 cells were pretreated for 1 h with protein kinase C inhbitors RO 31-8220 or Calphostin C, or IκBα inhibitor PDTC, with all other conditions pretreated with an equal-volume dimethylsulfoxide (DMSO) vehicle control. Cells were then stimulated with HBP and IL-6 levels were measured in the supernatant 24 h following stimulation. Groups were compared with the condition with HBP with no pretreatment (far left) using a one-way ANOVA followed by Dunns post-test for multiple comparisons. B, HK-2 cells were pretreated for 1 h with heparinase III (Hep. III) or Chondrotinase ABC (Chondro. ABC). Cells were then stimulated with HBP and IL-6 levels were measured in the supernatant 24 h following stimulation. Groups were compared to the condition with HBP with no pretreatment (far left) using a one-way ANOVA followed by Dunns post-test for multiple comparisons. Data are the mean, n = 3. IL-6 indicates interleukin-6; PKC, protein kinase C.

that both heparan sulfate and chondroitin/dermatan sulfate chains are involved.

HBP and renal inflammation in vivo

The effects of HBP on renal inflammation were investigated in a murine model. Plasma concentration of HBP at the end of the experiment was 400 ± 157 ng/mL in HBP-treated animals and 1.1 ± 1.5 ng/mL in controls. The kidneys were visualized histologically, revealing interstitial hemorrhage in mice treated with HBP that was not present in controls (Fig. 4A). HBP also significantly increased the amount of albumin in kidney sections, as visualized by immunohistochemical staining. This is suggestive of increased vascular leakage in the kidney (Fig. 4, A and B). This was further confirmed by the appearance of protein aggregates in the interstitial space, visualized by scanning electron microscopy (SEM) (Fig. 4A). While UFH seemed to decrease the amount of hemorrhage in the kidney visualized by histology and the amount of protein aggregation visualized by SEM, it did not significantly reduce the amount of albumin measured by immunohistochemistry. Changes observed after LPS administration were similar to those after HBP administration (Fig. 4A).

DISCUSSION

Our major findings were that elevated plasma HBP is associated with the presence and development of AKI in human septic shock, and that HBP induces inflammation in renal tubular cells *in vitro* and renal injury in a murine model, suggesting a role for HBP in the pathophysiology of sepsis-induced AKI. Finally, we showed that UFH and LMWHs block HBP-induced renal tubular cell inflammation, suggesting therapeutic potential for the prevention and/or treatment of sepsis-induced AKI.

The mechanism(s) leading to development and progression of AKI in septic shock are not fully understood and may involve vascular leakage, local renal cell inflammation, and cell cycle arrest (9). HBP has been shown to increase endothelial permeability (13), leading to lung damage in mice (14). Our *in vivo* study HBP induced hemorrhage and vascular leak in the kidney. Therefore, increased HBP may directly injure the kidneys by increasing permeability and renal edema, which may alter glomerular filtration and tubular function (9). Also, we describe for the first time that HBP induces an inflammatory response in renal tubular cells. Thus, HBP fulfills two out of three suggested mechanisms in the recently proposed unified theory of sepsis-induced AKI (9). Neutrophils may be culprits in AKI development and our data support a significant role for neutrophil-derived HBP in sepsis-induced AKI.

The concentration of HBP required for the *in vitro* effects is higher than median levels found in blood of sepsis patients with AKI. The majority of HBP in neutrophils (74%) is stored in azurophilic granules that are released after the neutrophils have infiltrated into the tissues (33). The interstitial space of the tissues is limited in volume and fluid is not circulated rapidly so the HBP concentration in the tissues is likely far higher than that in the blood. Therefore, it makes sense that the cells of the kidney would have a high threshold for HBP exposure compared with the levels found in the blood.

The cell-surface receptor for HBP on epithelial cells is unknown. HBP binds to GAG moieties of proteoglycans on the endothelium (27). Digestion of cell-surface GAGs prevents HBP binding to endothelial cells (11) and prevents HBP-induced endothelial permeability (14), but their role in renal tubular epithelial cells was unknown. We found that digestion of both chondroitin and heparan sulfate from the surface of renal tubular epithelial cells blocked HBP-induced IL-6 production, indicating that both heparan and chondroitin/dermatan

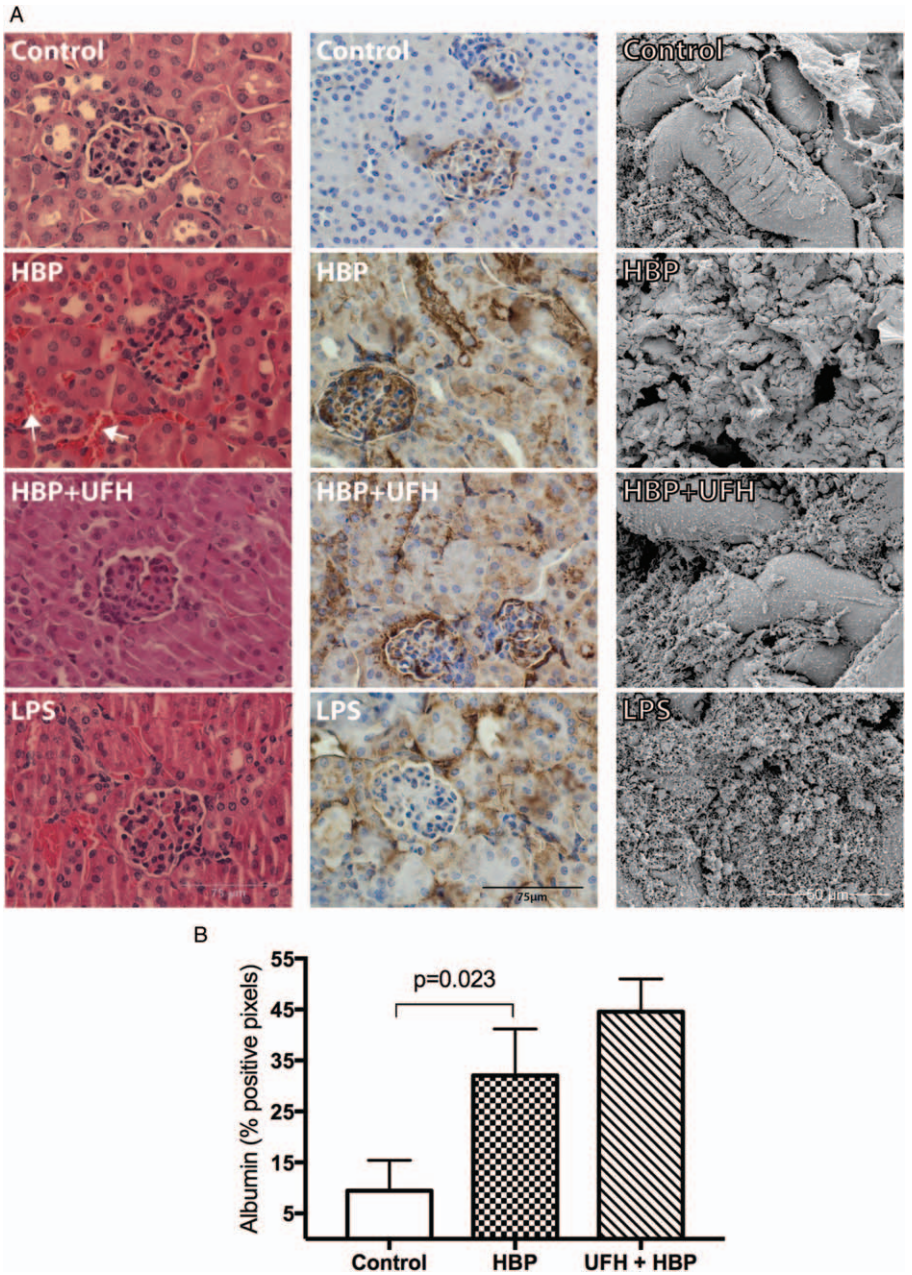

Fig. 4. **HBP induces hemorrhage and protein leakage in the kidneys.** Mice were injected with intravenous HBP and/or unfractionated heparin (UFH) followed by continuous infusion of HBP for 1 h. Controls received vehicle (0.9% normal saline). Mice injected with LPS served as a positive control. A, The kidneys were fixed and stained with hematoxylin and eosin (left) or stained with anti-albumin antibodies and visualized with 3,3-diaminobenzidine as the chromogen (middle) or analyzed by scanning electron microscopy (right). White arrows indicate areas of hemorrhage. B, The combined percentage of positive and high positive pixels was quantified in kidney sections stained with anti-albumin antibodies with 3,3-diaminobenzidine as the chromogen using the ICH Profiler plugin in Image J. Groups were compared using a one-way ANOVA followed by Dunns post-test for multiple comparisons. Data are the mean combined percentage of positive and high positive pixels, n = 3.

sulfate proteoglycans mediate HBP-induced renal cell inflammation. This also provides a mechanism for the action of heparin against HBP as heparin likely blocks GAG binding sites on HBP (34), preventing its association with cell surface GAGs. This may occur through electrostatic interactions, so other negatively charged molecules may also be capable of blocking HBP.

HBP activates PKC in corneal epithelial (35) and endothelial cells (36). We found that HBP-induced renal tubular cell inflammation is mediated by PKC and IκBα. IL-6 production is known to involve IκBα phosphorylation (37), and PKC activation in certain tissues (38). Thus, HBP likely acts through a cellular receptor that activates this pathway.

There are potentially important clinical implications of our study of UFH and LMWHs. Our results suggest that heparin(s) could be tested for efficacy and safety in prevention of sepsis-induced AKI. UFH and LMWHs at least partially attenuated HBP-induced renal vascular leakage *in vivo* and inflammation *in vitro*. These results add further rationale for the potential role of heparin derivatives in the treatment of septic shock (20). The

heparins used in this study are relatively inexpensive and readily available clinically. Further randomized, controlled trials of heparin(s) in septic shock are needed to determine their efficacy relative to the risk of hemorrhage.

The strengths of the current study are first, the large sample size of a well-characterized cohort of patients who had septic shock. Second, plasma samples were analyzed using a standardized, commercially available ELISA. Third, we tested the effects of HBP and inhibition by UFH both *in vivo* and *in vitro*.

Relative weaknesses of this study include the timing of plasma collection (median 18 h after development of septic shock). Because neutrophil-derived HBP likely is an early marker, measurement of HBP at 18 h could have limited the prognostic potential and increased false-negative findings. Assessment of changes in serial measurements of HBP could improve prognostic accuracy. The cohort was not ideal for the evaluation of HBP as an AKI biomarker; however, our results indicate that this should be validated in the future in a more suitable cohort. Also, we examined heparin administration only as a pretreatment *in vitro* or directly after HBP administration *in vivo*. The timing of heparin administration should be examined in future studies. Additionally, immortalized cells were used which may affect some cellular responses. Lastly, the consequences of HBP on renal function *in vivo* were not investigated.

CONCLUSIONS

Elevated levels of heparin-binding protein (HBP) were associated with the presence and progression of acute kidney injury (AKI) in septic shock patients. HBP is likely involved in the pathophysiology of AKI by inducing inflammation in renal tubular cells *in vitro* and hemorrhage and edema *in vivo*. UFH and LMWHs blocked HBP and provide greater rationale for future trials of heparin(s) in septic shock. Further clinical studies are needed to validate these findings and further define the mechanism(s) of HBP-induced kidney injury in septic shock.

ACKNOWLEDGMENTS

The authors thank Maria Baumgarten and Sandra Jovic for excellent technical assistance, and the Copenhagen University Core Facility for Integrated Microscopy (CFIM) for cutting edge electron microscopy environment.

REFERENCES

1. Ricci Z, Cruz D, Ronco C: The RIFLE criteria and mortality in acute kidney injury: a systematic review. *Kidney Int* 73(5):538–546, 2008.
2. Chertow GM, Burdick E, Honour M, Bonventre JV, Bates DW: Acute kidney injury, mortality, length of stay, and costs in hospitalized patients. *J Am Soc Nephrol* 16(11):3365–3370, 2005.
3. Linder A, Fjell C, Levin A, Walley KR, Russell JA, Boyd JH: Small acute increases in serum creatinine are associated with decreased long-term survival in the critically ill. *Am J Respir Crit Care Med* 189(9):1075–1081, 2014.
4. Kellum JA, Lameire N: Diagnosis, evaluation, and management of acute kidney injury: a KDIGO summary (Part 1). *Crit Care* 17(1):204, 2013.
5. Mishra J, Dent C, Tarabishi R, Mitsnefes MM, Ma Q, Kelly C, Ruff SM, Zahedi K, Shao M, Bean J, et al.: Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. *Lancet* 365(9466): 1231–1238, 2005.
6. Herget-Rosenthal S, Marggraf G, Husing J, Goring F, Pietruck F, Janssen O, Philipp T, Kribben A: Early detection of acute renal failure by serum cystatin C. *Kidney Int* 66(3):1115–1122, 2004.
7. Hall IE, Coca SG, Perazella MA, Eko UU, Luciano RL, Peter PR, Han WK, Parikh CR: Risk of poor outcomes with novel and traditional biomarkers at clinical AKI diagnosis. *Clin J Am Soc Nephrol* 6(12):2740–2749, 2011.
8. Hotchkiss RS, Karl IE: The pathophysiology and treatment of sepsis. *N Engl J Med* 348(2):138–150, 2003.
9. Gomez H, Ince C, De Backer D, Pickkers P, Payen D, Hotchkiss J, Kellum JA: A unified theory of sepsis-induced acute kidney injury: inflammation, microcirculatory dysfunction, bioenergetics, and the tubular cell adaptation to injury. *Shock* 41(1):3–11, 2014.
10. Soehnlein O, Lindbom L: Neutrophil-derived azurocidin alarms the immune system. *J Leukoc Biol* 85(3):344–351, 2009.
11. Soehnlein O, Xie X, Ulbrich H, Kenne E, Rotzius P, Flodgaard H, Eriksson EE, Lindbom L: Neutrophil-derived heparin-binding protein (HBP/CAP37) deposited on endothelium enhances monocyte arrest under flow conditions. *J Immunol* 174(10):6399–6405, 2005.
12. Herwald H, Cramer H, Mörgelin M, Russell W, Sollenberg U, Norrby-Teglund A, Flodgaard H, Lindbom L, Björck L: M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage. *Cell* 116(3):367–379, 2004.
13. Gautam N, Olofsson AM, Herwald H, Iversen LF, Lundgren-Akerlund E, Hedqvist P, Arfors KE, Flodgaard H, Lindbom L: Heparin-binding protein (HBP/CAP37): a missing link in neutrophil-evoked alteration of vascular permeability. *Nat Med* 7(10):1123–1127, 2001.
14. Bentzer P, Fisher J, Kong HJ, Morgelin M, Boyd JH, Walley KR, Russell JA, Linder A: Heparin-binding protein is important for vascular leak in sepsis. *Intensive Care Med Exp* 4(1):33, 2016.
15. Linder A, Christensson B, Herwald H, Björck L, Åkesson P: Heparin-binding protein: an early marker of circulatory failure in sepsis. *Clin Infect Dis* 49(7):1044–1050, 2009.
16. Linder A, Arnold R, Boyd JH, Zindovic M, Zindovic I, Lange A, Paulsson M, Nyberg P, Russell JA, Pritchard D, et al.: Heparin-binding protein measurement improves the prediction of severe infection with organ dysfunction in the emergency department. *Crit Care Med* 43(11):2378–2386, 2015.
17. Linder A, Åkesson P, Inghammar M, Treutiger CJ, Linner A, Sunden-Cullberg J: Elevated plasma levels of heparin-binding protein in intensive care unit patients with severe sepsis and septic shock. *Crit Care* 16(3):R90, 2012.
18. Cornet AD, Smit EG, Beishuizen A, Groeneveld AB: The role of heparin and allied compounds in the treatment of sepsis. *Thromb Haemost* 98(3):579–586, 2007.
19. Ostergaard E, Flodgaard H: A neutrophil-derived proteolytic inactive elastase homologue (hHBP) mediates reversible contraction of fibroblasts and endothelial cell monolayers and stimulates monocyte survival and thrombospondin secretion. *J Leukoc Biol* 51(4):316–323, 1992.
20. Zarychanski R, Doucette S, Fergusson D, Roberts D, Houston DS, Sharma S, Gulati H, Kumar A: Early intravenous unfractionated heparin and mortality in septic shock. *Crit Care Med* 36(11):2973–2979, 2008.
21. Slofstra SH, van 't Veer C, Buurman WA, Reitsma PH, ten Cate H, Spek CA: Low molecular weight heparin attenuates multiple organ failure in a murine model of disseminated intravascular coagulation. *Crit Care Med* 33(6):1365–1370, 2005.
22. Russell JA, Walley KR, Singer J, Gordon AC, Hebert PC, Cooper DJ, Holmes CL, Mehta S, Granton JT, Storms MM, et al.: Vasopressin versus norepinephrine infusion in patients with septic shock. *N Engl J Med* 358(9):877–887, 2008.
23. Russell JA, Fjell C, Hsu JL, Lee T, Boyd J, Thair S, Singer J, Patterson AJ, Walley KR: Vasopressin compared with norepinephrine augments the decline of plasma cytokine levels in septic shock. *Am J Respir Crit Care Med* 188(3):356–364, 2013.
24. Fjell CD, Thair S, Hsu JL, Walley KR, Russell JA, Boyd J: Cytokines and signaling molecules predict clinical outcomes in sepsis. *PLoS One* 8(11): e79207, 2013.
25. Hirsh J, Anand SS, Halperin JL, Fuster V, American Heart A: Guide to anticoagulant therapy: Heparin: a statement for healthcare professionals from the American Heart Association. *Circulation* 103(24):2994–3018, 2001.
26. Garcia DA, Baglin TP, Weitz JI, Samama MM, American College of Chest P: Parenteral anticoagulants: antithrombotic therapy and prevention of thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest* 141(2 suppl):e24S–43S, 2012.
27. Olofsson AM, Vestberg M, Herwald H, Rygaard J, David G, Arfors KE, Linde V, Flodgaard H, Dedio J, Muller-Esterl W, et al.: Heparin-binding protein targeted to mitochondrial compartments protects endothelial cells from apoptosis. *J Clin Invest* 104(7):885–894, 1999.
28. Dull RO, Dinavahi R, Schwartz L, Humphries DE, Berry D, Sasisekharan R, Garcia JG: Lung endothelial heparan sulfates mediate cationic peptide-induced barrier dysfunction: a new role for the glycocalyx. *Am J Physiol Lung Cell Mol Physiol* 285(5):L986–L995, 2003.

29. Ding R, Zhao D, Guo R, Zhang Z, Ma X: Treatment with unfractionated heparin attenuates coagulation and inflammation in endotoxemic mice. *Thromb Res* 128(6):e160–e165, 2011.
30. Bhargava R, Altmann CJ, Andres-Hernando A, Webb RG, Okamura K, Yang Y, Falk S, Schmidt EP, Faubel S: Acute lung injury and acute kidney injury are established by four hours in experimental sepsis and are improved with pre, but not post, sepsis administration of TNF-alpha antibodies. *PLoS One* 8(11):e79037, 2013.
31. Chappell D, Jacob M, Rehm M, Stoeckelhuber M, Welsch U, Conzen P, Becker BF: Heparinase selectively sheds heparan sulphate from the endothelial glycocalyx. *Biol Chem* 389(1):79–82, 2008.
32. Hamai A, Hashimoto N, Mochizuki H, Kato F, Makiguchi Y, Horie K, Suzuki S: Two distinct chondroitin sulfate ABC lyases. An endoeliminase yielding tetrasaccharides and an exoeliminase preferentially acting on oligosaccharides. *J Biol Chem* 272(14):9123–9130, 1997.
33. Tapper H, Karlsson A, Mörgelin M, Flodgaard H, Herwald H: Secretion of heparin-binding protein from human neutrophils is determined by its localization in azurophilic granules and secretory vesicles. *Blood* 99(5): 1785–1793, 2002.
34. McCabe D, Cukierman T, Gabay JE: Basic residues in azurocidin/HBP contribute to both heparin binding and antimicrobial activity. *J Biol Chem* 277(30):27477–27488, 2002.
35. Griffith GL, Russell RA, Kasus-Jacobi A, Thavathiru E, Gonzalez ML, Logan S, Pereira HA: CAP37 activation of PKC promotes human corneal epithelial cell chemotaxis. *Invest Ophthalmol Vis Sci* 54(10):6712–6723, 2013.
36. Pereira HA, Moore P, Grammas P: CAP37, a neutrophil granule-derived protein stimulates protein kinase C activity in endothelial cells. *J Leukoc Biol* 60(3):415–422, 1996.
37. Tak PP, Firestein GS: NF-kappaB: a key role in inflammatory diseases. *J Clin Invest* 107(1):7–11, 2001.
38. Gross V, Zhang B, Geng Y, Villiger PM, Lotz M: Regulation of interleukin-6 (IL-6) expression: evidence for a tissue-specific role of protein kinase C. *J Clin Immunol* 13(5):310–320, 1993.